REMARKS

Upon entry of the foregoing amendments, Claims 144-153 are pending. Applicants have cancelled Claims 128-143 without prejudice to, or disclaimer of, the subject matter therein. Applicants maintain that the cancellation of a claim makes no admission as to its patentability, and reserve the right to pursue the subject matter of any cancelled claim in this or any other patent application.

Applicants have added new Claims 144-153. The amendments add no new matter and are fully supported by the specification and claims as originally filed. Support for the amendments can be found, for example, in the abstract, paragraphs [0018], [0031], [0034], [0037], [0043], [0044], [0061], [0063], [0064], [0066], [0103], [0108], and elsewhere throughout the specification.

Claims 128-143 were rejected in the final Office Action mailed January 7, 2010. Inasmuch as the rejections relate to the claims presented herein for examination, Applicants respond to the specific rejections set forth in the final Office Action. For the reasons set forth below, Applicants respectfully traverse.

Rejection Under 35 U.S.C. § 112, second paragraph

The Examiner rejected Claims 128-143 as allegedly failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. The Examiner states that the metes and bounds of the terms "substantially disaggregated," "prolonged survival," and "improved vascularity," are unclear, and render the claims indefinite.

While Applicants do not acquiesce to the Examiner's position, Applicants have removed the allegedly offending terms from the claims presented herein for examination. As such, the presently pending claims do not raise the issues raised by the Examiner in the final Office Action under 35 U.S.C. § 112, second paragraph.

Rejection Under 35 U.S.C. § 102(e) – Katz et al.

The Examiner rejected Claims 128-143 as allegedly being anticipated by U.S. Patent No. 6,777,231 to Katz et al. ("Katz"). According to the Examiner, Katz teaches "adipose tissue that contains adipose derived stem cells." The Examiner asserts that the amount of added cells

required in Claims 128-143 is "nearly negligible. . .and even where higher amounts are claimed, the resultant mixture would still. . .read on a typical adipose tissue sample." (Office Action, 3-4). The Examiner states that "a 'concentrated' mixture produced by applicant's method will likely contain the same amount of adipose-derived cells as another mixture which is simply naturally more concentrated with the designated cells." (Office Action, 4). As such, the Examiner concludes that Katz anticipates Applicants' composition.

Katz describes a composition of adipose derived stem cells "substantially free of other cell types ... and extracellular matrix material," that is derived from excised adipose tissue (Katz, Column 2, lines 28-32). In other words, in Katz, one unit of adipose tissue removed from a patient is processed to yield a composition consisting essentially of stem cells. To arrive at this composition, Katz discards the other cells and the extracellular matrix materials found in adipose tissue. (See, e.g., Katz, Col. 3, line 19-Col. 4, line 6). As discussed below, neither the excised adipose tissue of Katz, nor the Katz composition that consists essentially of stem cells, meets the limitations of Applicants' claimed compositions.

New independent Claim 144 is drawn to an autologous adipose tissue implant comprising: (1) a first portion of intact (non-disaggregated) adipose tissue removed from a patient, mixed with (2) a concentrated population of adipose-derived cells that comprises stem cells, wherein the population of adipose-derived cells comprising stem cells is concentrated from a second portion of adipose tissue removed from the same patient, and wherein the stem cell concentration in the concentrated population of adipose-derived cells that comprises stem cells is more than 1.7×10^5 cells/ml. New independent Claim 153 is drawn to an autologous adipose tissue implant, comprising: (1) a first portion of intact (non-disaggregated) adipose tissue comprising connective tissue removed from a patient, mixed with (2) a concentrated population of adipose-derived cells that comprises stem cells, wherein the population of adipose-derived cells comprising stem cells is concentrated from a second portion of adipose tissue that is removed from the same patient and enzymatically treated, wherein the stem cell concentration in the concentrated population of adipose-derived cells that comprises stem cells is more than 1.7×10^5 cells/ml.

Thus, Applicants' claimed compositions are formed from two components. To arrive at Applicants' claimed compositions, adipose tissue is removed from a patient. One portion of the

adipose tissue is left intact (first portion) and the other portion (second portion) is processed to yield a concentrated population of stem cells and other cells found in adipose tissue. As such, by definition, the concentrated population of cells comprising stem cells in the second portion is greater than the concentration of stem cells in first portion of excised/unprocessed adipose tissue. By mixing the concentrated population of adipose-derived cells comprising stem cells with intact adipose tissue from the same individual, the resultant autologous fat graft, by definition, has a higher concentration of stem cells than native adipose tissue. As such, any teaching of excised adipose tissue in Katz fails to anticipate, either expressly or inherently, Applicants' presently claimed compositions.

The cells and cell populations described in Katz also fail to meet the limitations of Applicants' claimed compositions. Specifically, Applicants note that Katz removes extracellular matrix material from the excised adipose tissue. By contrast, Applicants' presently claimed composition includes an intact (first) portion of adipose tissue that retains the extracellular matrix material that is removed from the Katz composition.

For the foregoing reasons, neither the adipose tissue nor the compositions described in Katz anticipate the adipose tissue implant of Claim 144 and 153, and, by extension, dependent Claims 145-152, which necessarily includes a greater amount of adipose-derived stem cells than a native adipose tissue sample from the same patient.

In view of the foregoing, Applicants respectfully submit that the present claims are not anticipated under 35 U.S.C. § 102(e) by Katz.

Rejection Under 35 U.S.C. § 102(b) – Hu et al.

The Examiner rejected Claims 128-143 as allegedly being anticipated by U.S. Patent No. 5,744,360 to Hu et al. ("Hu"). According to the Examiner, Hu discloses adipose tissue which has been excised from the body, and which "inherently contains adipose derived stem cells." (Office Action, 4). The Examiner relies upon the same reasoning used in the assertion that Katz anticipates Applicants' claims to arrive at the conclusion that the adipose tissue sample described in Hu anticipates Applicants' claimed compositions.

Hu describes a device and method for harvesting adipose tissue, in order to increase the number of viable microvascular endothelial cells that can be obtained from the tissue upon

further processing. Specifically, Hu describes a device with certain features, *i.e.*, a cannula with tissue cutting edges, a means for creating a suction force, and a homogenizing member to disrupt the connective matrix of the excised adipose tissue, that allegedly preserve the number of viable microvasular endothelial cells in excised adipose tissue, which can then be further processed. In fact, Hu states that the isolated adipose tissue obtained in the device is either "processed further or stored for later use." (Hu, Col. 10, lines 39-40).

Hu is completely silent regarding a composition comprising an adipose tissue implant. The excised adipose tissue in Hu does not contain both components of Applicants' presently claimed compositions. Specifically, the excised adipose tissue of Hu is not mixed with a concentrated population of adipose-derived cells comprising stem cells, as required by Applicants' claimed compositions. As such, for the same reasons as set forth with respect to Katz, the excised adipose tissue in Hu will necessarily differ from Applicants' claimed compositions, in that Applicants' presently claimed compositions necessarily have a higher concentration of adipose-derived cells comprising stem cells than excised adipose tissue.

The processed adipose tissue/cell populations mentioned in Hu also cannot anticipate Applicants' presently claimed compositions. Hu mentions further processing excised adipose tissue "using digestion and other procedures" in order to separate the desired cellular component from the adipose tissue. (Hu, Col. 10, lines 39-49). Hu never mentions a concentrated population of adipose-derived cells comprising stem cells, and also does not mention mixing such a concentrated population of adipose-derived cells comprising stem cells with intact, non-disaggregated adipose tissue, as required by Applicants' present claims. As such, the processed adipose tissue/cell populations described in Hu cannot anticipate Applicants' compositions since the cell populations derived from adipose tissue are not provided in a composition that also includes intact, non-disaggregated adipose tissue.

For the reasons set forth above, Applicants respectfully submit that Hu cannot anticipate Applicants' present claims under 35 U.S.C. § 102(b). .

Rejection Under 35 U.S.C. § 103(a) – Katz et al. and Peterson et al.

The Examiner rejected Claims 128-143 as allegedly being unpatentably obvious over Katz et al. and U.S. Patent No. 6,200,606 to Peterson et al. ("Peterson"). According to the

Examiner, both Katz and Peterson teach compositions comprising adipose-derived stem cells that are substantially free of other cells and tissues. The Examiner states that the only difference between Applicants' compositions and the compositions disclosed in Katz and Peterson is "a matter of the concentration of the cells and tissues contained therein." (Office Action, 6). The Examiner concludes that Applicants' compositions are *prima facie* obvious, in the absence of evidence indicating that the concentration of the tissue or the cell populations comprising adipose-derived stem cells is critical.

The presently claimed compositions are not *prima facie* obvious in view of the Katz and Peterson references, which disclose substantially isolated adipose-derived stem cells. Specifically, when considered as a whole, the teachings of Katz and Peterson not only fail to render all limitations of Applicants' present claims obvious, but further lead the skilled artisan away from Applicants' claimed compositions. Additionally, as discussed herein, Applicants' evidence and data demonstrate that the presently claimed tissue implants provide unexpected and unpredictable benefits over the tissue implants known as of Applicants' effective filing date (*e.g.*, either unsupplemented adipose tissue, or isolated populations of disaggregated adipose-derived cells). In other words, the evidence and data demonstrate that supplementing intact, non-disaggregated adipose tissue with a concentrated population of adipose-derived cells comprising stem cells, which leads to a composition that has a higher concentration of adipose-derived stem cells than adipose tissue alone, results in the unexpectedly beneficial characteristics of the presently claimed supplemented implant.

In contrast to Applicants' claimed grafts and implants, the compositions of both Katz and Peterson devoid of any intact, non-disaggregated adipose tissue. In fact, both Katz and Peterson teach away from Applicants' claimed grafts and implants, as both references teach the desirability of complete disaggregation and separation of stem cells from adipose tissue matrix, e.g., unprocessed adipose tissue. Katz states that it is advantageous to separate the stem cells present in the intact adipose tissue from all other cells and extracellular matrix material present in intact adipose-tissue:

> preferably, the stem cell is substantially free of other cell types (*e.g.* adipocytes, red blood cells, other stromal cells, etc.) and extracellular matrix material; more preferably, the stem cell is completely free of such other cell types and matrix material. (Katz, Col. 2, lines 27-32, emphasis added)

The teachings of Katz would lead the skilled artisan away from any graft or implant similar to Applicants' presently claimed grafts, which comprise intact, non-disaggregated adipose tissue, mixed with a concentrated population of adipose-derived cells that comprises stem cells, wherein the population of adipose-derived cells comprising stem cells is concentrated from a second portion of adipose tissue removed from the same patient, and wherein the stem cell concentration in the concentrated population of adipose-derived cells that comprises stem cells is more that 1.7×10^5 cells/ml.

Likewise, Peterson teaches the desirability of completely isolating precursor cells from other components of adipose tissue. Specifically, Peterson teaches two embodiments that involve the use of adipose tissue as a source of precursor cells. Both embodiments require a step of disaggregating the tissue to achieve a single-cell suspension, which is then further processed to isolate the cells of interest. Peterson states that it is necessary to fully disaggregate and further process adipose tissue. (Peterson, Col. 10, lines 5-8, Col. 10, lines 15-20). Accordingly, the skilled artisan would be lead away from a graft or implant similar to Applicants' presently claimed grafts.

In addition to the fact that the cited references teach away from Applicants' presently claimed compositions, Applicants note that the presently claimed grafts provide superior and unexpected benefits over the compositions disclosed in Katz and Peterson.

Numerous articles from peer-reviewed journals confirm that the cell populations described in Katz and Peterson, are not suitable as autologous tissue grafts, either alone, or when the cells are seeded onto various lattices, matrices, or scaffolds. (See, Declaration of John K. Fraser, Ph.D., Submitted April 9, 2008, and Exhibits annexed thereto). Implants comprising disaggregated adipose-derived stem cells seeded on a lattice, scaffold, or matrix (such as the types mentioned in Katz and Peterson) are unsuitable because they are resorbed over time. Furthermore, the cell-seeded scaffolds and matrices do not have the characteristics of native soft tissue, rendering them unsuitable for a large number of implants. (See, Fraser Decl., ¶¶ 5-7). Further, numerous articles also demonstrate that excised adipose tissue alone (*i.e.*, adipose tissue that has not been supplemented with a concentrated population of adipose-derived cells) is also unsuitable. By way of example, Tabata et al., discussed in Fraser Declaration, and resubmitted

herewith as **Exhibit A**, state that transplants of autologous adipose tissue alone is known to become necrotic over time, and become absorbed. (See, Tabata et al., p. 279, first paragraph).[1]

By mixing non-disaggregated adipose tissue with a concentrated population of cells comprising adipose-derived stem cells in order to achieve a higher concentration of stem cells than in isolated adipose tissue, Applicants are able to achieve unexpected benefits over adipose-derived tissue implants known in the art, which concerned either isolated adipose tissue alone or disaggregated adipose-derived stem cell containing populations alone or with synthetic matrices or scaffolds.

As previously set forth during the prosecution of this application, Applicants have shown that graft weight and vascularity of adipose tissue implants are improved by supplementing unprocessed adipose tissue with isolated preparations of adipose-derived cells comprising stem cells and progenitor cells. (See, Specification, Example 1). Applicants have provided additional data that confirms the data set forth in the specification regarding the unexpected benefits of the presently claimed compositions. (See, Fraser Declaration). The results of the studies in Example 1 of the instant specification were confirmed in a human clinical trial, wherein twenty-one (21) female patients underwent 25 stem cell augmented reconstructions. Adipose tissue was harvested by lipoaspiration, divided into two equal portions; one portion, referred to as "Fat A", was reserved for processing in the cell processing system described in the instant patent application to extract, wash and concentrate adipose derived stem cells; the other portion, "Fat B", was used as the primary filler material. Concurrent to the processing of "Fat A", "Fat B" was irrigated to remove any blood, and the remaining adipose tissue, which had been fragmented into numerous 2 - 5 mm fragments by the lipoaspiration procedure, was enriched with concentrated adipose-derived stem cells out of the cell processing device by gentle mixing immediately prior to the autologous transplantation procedure. Subsequently, the autologous adipose derived stem and regenerative cell (ADRC) enhanced adipose tissue graft was provided to the patient and the grafts remained intact for the longest time period analyzed (eighteen months).

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[1] Tabata et al. states: Autografting of fat pads has a long history in plastic and reconstructive surgery for augmentation of lost soft tissues. It has been reported that autologous adipose tissues, such as fat grafts of a few millimeters in size and semiliquid, were transplanted to depressed regions or scares in the breast and facial areas. Despite the enthusiasm for such the free-fat autografting, however, researches have been disappointed by progressive absorption of the tissue graft with time. In the examination by microscopy of free-fat autografts removed, necrotic adipocytes were observed and replaced by host fibrous tissue in most areas whereas the transplanted fat cells were hardly proliferated. (Tabata, p. 279, first paragraph).

In addition to the clinical trials with humans, the unexpected benefits of Applicants' tissue grafts were demonstrated in experiments using a murine fat transplantation model, as described in Applicants' Amendment and Response filed August 7, 2008. Applicants' experimental data from the murine model confirmed the data provided in the specification, namely that the supplementation of unprocessed, non-disaggregated adipose tissue with a concentrated population of cells comprising adipose-derived stem cells results in improved graft retention, increased graft weight, and improved vascularity, survival of the graft. In other words, the mixture of unprocessed, non-disaggregated adipose tissue with a concentrated population of adipose-derived cells comprising stem cells is evidence that having a concentration of adipose-derived cells comprising stem cells that is greater than excised adipose tissue, provides unexpected benefits.

The unexpected benefits of Applicants' presently claimed compositions could not have been predicted given the state of the art, which teaches using unprocessed adipose tissue as a graft, or that uses adipose tissue that has not been supplemented with a concentrated population of cells comprising adipose-derived stem cells. In view of the state of the art, as described in Katz and Peterson, and further evidenced by the Fraser Declaration, as well as the unexpected benefits attributable to Applicants' discovery regarding the supplementation of unprocessed, non-disaggregated tissue, Applicants respectfully request that the rejections under 35 U.S.C. § 103(a) be withdrawn.

No Disclaimers or Disavowals

Although the present communication may include alterations to the application or claims, or characterizations of claim scope or referenced art, Applicant is not conceding in this application that previously pending claims are not patentable over the cited references. Rather, any alterations or characterizations are being made to facilitate expeditious prosecution of this application. Applicant reserves the right to pursue at a later date any previously pending or other broader or narrower claims that capture any subject matter supported by the present disclosure, including subject matter found to be specifically disclaimed herein or by any prior prosecution. Accordingly, reviewers of this or any parent, child or related prosecution history shall not

reasonably infer that Applicant has made any disclaimers or disavowals of any subject matter supported by the present application.

CONCLUSION

In view of the above amendments and remarks, Applicants respectfully maintain that the claims are patentable and request that they be passed to issue. Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: July 1, 2010

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EXHIBIT A

TISSUE ENGINEERING
Volume 6, Number 3, 2000
Mary Ann Liebert, Inc.

De Novo Formation of Adipose Tissue by Controlled Release of Basic Fibroblast Growth Factor

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ABSTRACT

***De novo* adipogenesis at the implanted site of a basement membrane extract (Matrigel) was induced through controlled release of basic fibroblast growth factor (bFGF). bFGF was incorporated into biodegradable gelatin microspheres for its controlled release. When the mixture of Matrigel and bFGF-incorporated gelatin microspheres was implanted subcutaneously into the back of mice, a clearly visible fat pad was formed at the implanted site 6 weeks later. Histologic examination revealed that the *de novo* formation of adipose tissue accompanied with angiogenesis was observed in the implanted Matrigel at bFGF doses of 0.01, 0.1, and 1 μg/site, the lower and higher doses being less effective. The *de novo* formation induced by the bFGF-incorporated microspheres was significantly higher than that induced by free bFGF of the same dose. The mRNA of a lipogenesis marker protein, glycerophosphate dehydrogenase, was detected in the formed adipose tissues, biochemically indicating *de novo* adipogenesis. Free bFGF, the bFGF-incorporated gelatin microspheres, or Marigel alone and bFGF-free gelatin microspheres with or without Matrigel did not induce formation of adipose tissue. This *de novo* adipogenesis by mixture of Matrigel and the bFGF-incorporated gelatin microspheres will provide a new idea for tissue engineering of adipose tissue.**

INTRODUCTION

AUTOGRAFTING OF FAT PADS has a long history in plastic and reconstructive surgery for augmentation of lost soft tissues.[1] It has been reported that autologous adipose tissues, such as fat grafts of a few millimeters in size and semiliquid, were transplanted to depressed regions or scars in the breast and facial areas.[2,3] Despite the enthusiasm for such the free-fat autografting, however, researchers have been disappointed by progressive absorption of the tissue graft with time.[4–8] In the examination by microscopy of free-fat autografts removed, necrotic adipocytes were observed and replaced by host fibrous tissue in most areas whereas the transplanted fat cells were hardly proliferated.

It is generally recognized from recent research in cell biology that adipocyte linkage derives from mul-

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tipotential mesenchymal stem cells with the capacity to differentiate into mesodermal cells, *e.g.*, osteocytes, chondrocytes, adipocytes, and myocytes.[9] These stem cells are morphologically and biochemically converted to matured adipocytes (fat cells) by way of adipose precursor cells. Among the precursor cells are preadipocytes, which have committed or determined to become fat cells and are included in interstitial cells having fibroblast-like morphology.[10]

There are two possible ways based on tissue engineering to induce *de novo* adipogenesis. One method is to carry the preadipocytes in a body site to be induced. It is reported that a preadipocyte cell line induced formation of fat tissue after subcutaneous injection to nude mice.[11] Patrick *et al.* have demonstrated that formation of adipose tissue in the rat subcutis by use of porous discs of poly(lactic-co-glycolic) acid seeded with autologously isolated preadipocytes.[12] The other method is to induce formation of adipose tissue from preadipocytes originally existing in the body. If one can provide a microenvironment suitable for cell proliferation and differentiation, *de novo* formation of adipose tissue can be expected without exogenous adipocytes being transplanted. Recently, Kawaguchi *et al.* have demonstrated that *de novo* adipogenesis in the mouse subcutis could be achieved only by injection of the simple mixture of basic fibroblast growth factor (bFGF) and an extract of basement membrane protein (Matrigel).[13] It is reported that mixing with Matrigel enabled bFGF to promote the vascular response.[14] Thus, it seems reasonable to suppose that such development of a vascular supply is essential for generation and maintainance of the adipose tissue.

It is known that some growth factors promote vascularization. Among the growth factors used to induce capillary formation are acidic FGF, bFGF, platelet-derived growth factor, and vascular endothelial growth factor.[15–18] However, if these growth factors are applied in solution form, one cannot always exhibit their full angiogenic capability because of their *in vivo* short half-life periods. This implies that controlled release of growth factors is needed for effective vascularization. Recently, we have succeeded in releasing biologically active bFGF from a biodegradable hydrogel composed of "acidic" gelatin, which is able to form a polyion complex with "basic" bFGF.[19] *In vivo* experiments revealed that the time profile of bFGF retention in the gelatin hydrogels was in good accordance with that of their degradation.[20] This indicates that the bFGF molecule ionically interacting with the acidic gelatin is released from the hydrogel as the cross-linked gelatin became water soluble with *in vivo* degradation. bFGF-incorporated gelatin hydrogels of disc and microsphere types showed an enhanced angiogenetic effect, in marked contrast to free bFGF.[19,20–23]

The objective of the present study was to investigate the effect of gelatin microphores for bFGF release on *de novo* formation of adipose tissue in Matrigel. Following subcutaneous implantation of Matrigel mixed with the bFGF-incorporated gelatin microphores into the backs of mice, *de novo* adipogenesis was evaluated in terms of histologic and biochemical viewpoints and compared with that of Matrigel mixed with bFGF in solution form. We examine the effect of bFGF dose on the formation of adipose tissue.

MATERIALS AND METHODS

Materials

An aqueous solution of human recombinant bFGF with an isoelectric point (IEP) of 9.6 (10 mg/mL) was kindly supplied by Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan). A gelatin sample with an IEP of 5.0 (Nitta Gelatin Co., Osaka, Japan) was extracted from bovine bone (type I collagen) with an alkaline process. Matrigel® basement membrane matrix of growth factor reduced type (Matrigel, Lot# 911947, Becton Dickinson Labware, Bedford, MA) was used here to minimize the effect of growth factor as much as possible. Glutaraldehyde (GA), glycine, and other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan) and used without further purification.

Preparation of bFGF-incorporated gelatin microspheres

Gelatin microspheres were prepared through GA crosslinking of gelatin aqueous solution in an emulsion state as reported previously.[23] Immediately after mixing 25 μL of GA aqueous solution (25 wt%) with 10

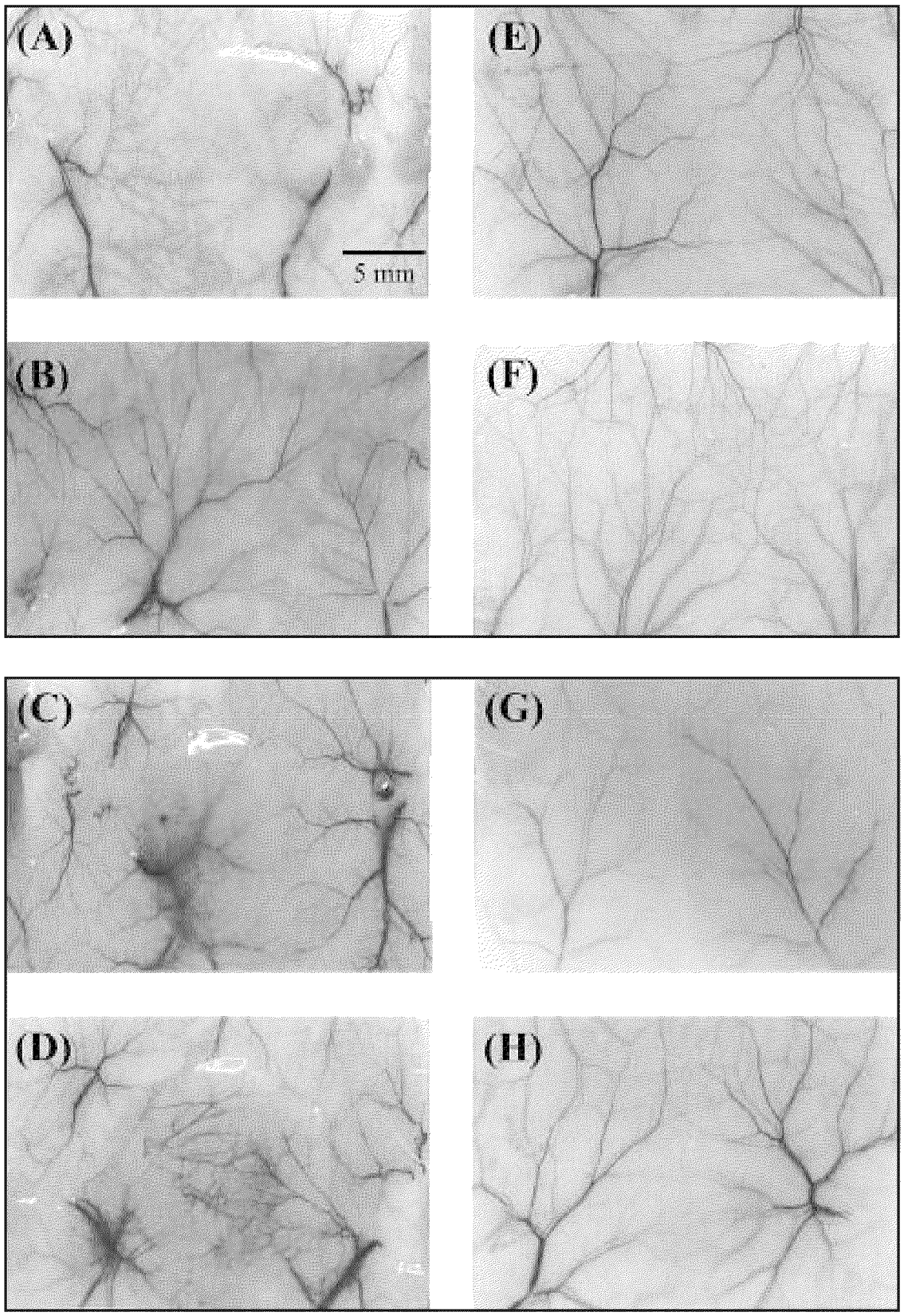


FIG. 1. Tissue appearance of mouse subcutis 6 weeks after treatment of PBS (**A,E**), bFGF-free, empty gelatin microspheres (**B,F**), 0.1 μg of free bFGF (**C,G**), and gelatin microspheres incorporating 0.1 μg of bFGF (**D,H**) with (**A–D**) or without Matrigel (**E–H**).

mL of 10 wt% gelatin aqueous solution preheated at 40°C, the mixed aqueous solution was added dropwise to 375 mL of olive oil with stirring at 420 rpm and 40°C to obtain a W/O emulsion. Stirring was continued for 24 h at 25°C to chemically crosslink the gelatin. After addition of 100 mL of acetone to the reaction mixture, the resulting microspheres were collected by centrifugation (4°C, 3000 rpm, 5 min) and washed five times with acetone by centrifugation. The washed microspheres were placed in 100 mL of 100 mM glycine aqueous solution containing Tween 80 (0.1 wt%), followed by agitation at 37°C for 1 h to block residual aldehyde groups of unreacted GA. Then, the crosslinked microspheres were twice washed with double-distilled water (DDW) by centrifugation, freeze-dried, and sterilized with ethylene oxide gas. The water content of the gelatin microspheres was 95 vol%, when calculated from the microsphere volume before and after swelling in phosphate-buffered saline solution (PBS, pH 7.4) for 24 h at 37°C. The microsphere diameter was measured by viewing at least 100 microspheres with a light microscope and found to range from 60 to 130 μm in the state of PBS swelling.

The original bFGF solution was diluted with DDW to adjust the solution concentration. The aqueous solution containing 0.001, 0.01, 0.1, 1.0, and 10 μg of bFGF (10 μL) as dropped onto 2 mg of freeze-dried gelatin microspheres for impregnation of bFGF into the microspheres. The bFGF solution was completely sorbed into the microspheres by swelling at 25°C for 1 h, because the solution volume was less than that theoretically required for the equilibrated swelling of microspheres. Similarly, empty gelatin microspheres without bFGF were prepared using DDW as the solution to add.

An animal experiment revealed that the gelatin microspheres used were degraded with time in the back subcutis of mice and disappeared 3 weeks later.[23] No influence of bFGF incorporation on the time profile microsphere degradation was observed. In this release system, because the bFGF release is governed by microsphere biodegradation,[20] bFGF is released from the microspheres over 3 weeks.

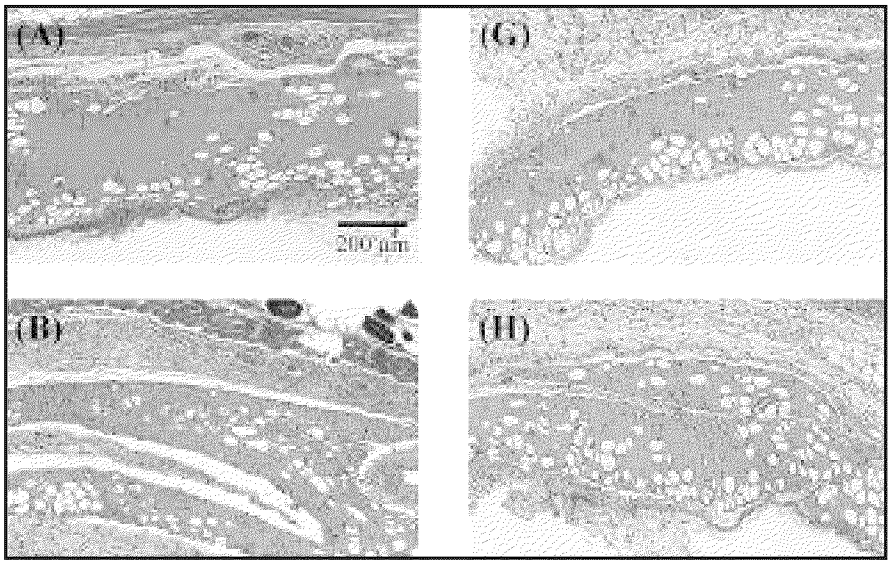
In vivo *experiments*

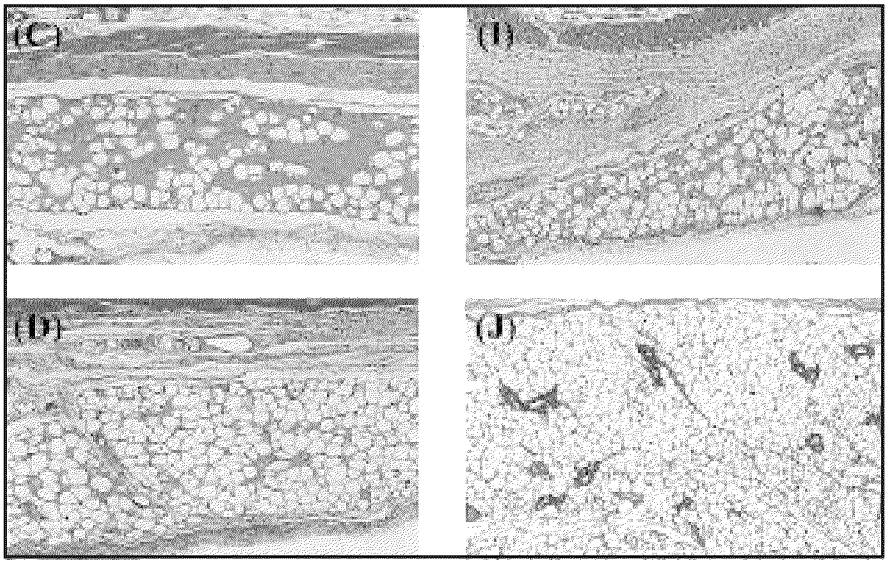
The gelatin microspheres (2 mg) swollen with aqueous solution with or without bFGF were homogeneously mixed with 100 μL of Matrigel precooled on ice. The mixture was left for 1 h at 37°C to allow Matrigel to form a hydrogel. As controls, 10 μL of aqueous solutions containing 0.001, 0.01, 0.1, 1.0, and 10 μg of bFGF and PBS were similarly mixed with Matrigel.

Under anesthesia, the mixture of Matrigel with bFGF-incorporated gelatin microspheres or other agents was carefully implanted into the back subcutis of female BALB/c mice (6 weeks old; Shimizu Laboratory Supply, Kyoto, Japan) 1.5 cm apart from the tail root at the body center. The materials used for Matrigel mixing were PBS, empty gelatin microspheres, five doses of free bFGF, and gelatin microspheres incorporating five doses of bFGF. In addition, the similar experiment was performed for the above 12 groups in the absence of Matrigel; PBS, empty gelatin microspheres, free bFGF, and gelatin microspheres incorporating bFGF alone were injected subcutaneously. Each experimental group was composed of 6 mice. The samples were carefully implanted or injected into the subcutaneous site free of originally existing adipose tissue. At 6 weeks post-treatment, the mice were sacrificed by an overdose injection of anesthetic and the skin including the implanted or injected site (2×2 cm^2) was carefully taken off for the subsequent biological examinations. Photographs of the skin flaps were taken to record tissue appearance around the treated site.

De novo formation of adipose tissue at the implanted or injected site was assessed in terms of histologic and biochemical parameters. Of the six skin flaps, three flaps were randomly selected for histological evaluation. The skin flaps were cut at the central portion of implanted or injected site by a scalpel. One cut of the skin was fixed with 10% neutralized formalin solution, embedded in paraffin, and sectioned (2 mm in thickness), followed by staining with hematoxylin and eosin (HE). The other skin cut was embedded in O.C.T. compound, TISSUE-TEK® (4583, Miles Inc., Elkhart, IN), cryosectioned, and stained with Sudan III. Photomicrographs of three cross sections from 3 different mice were taken at different magnifications

FIG. 2. Histologic sections of mouse subcutis 6 weeks after implantation of Matrigel mixed with 0 **(A)**, 0.001 **(B)**, 0.01 **(C)**, 0.1 **(D)**, 1.0 **(E)**, and 10 μg **(F)** of free bFGF or gelatin microspheres incorporating 0 **(G)**, 0.001 **(H)**, 0.01 **(I)**, 0.1 **(J)**, 1.0 **(K)**, and 10 μg **(L)** of bFGF. (Magnification: ×100, HE staining.)


(A)
200 μm
(G)
(B)
(H)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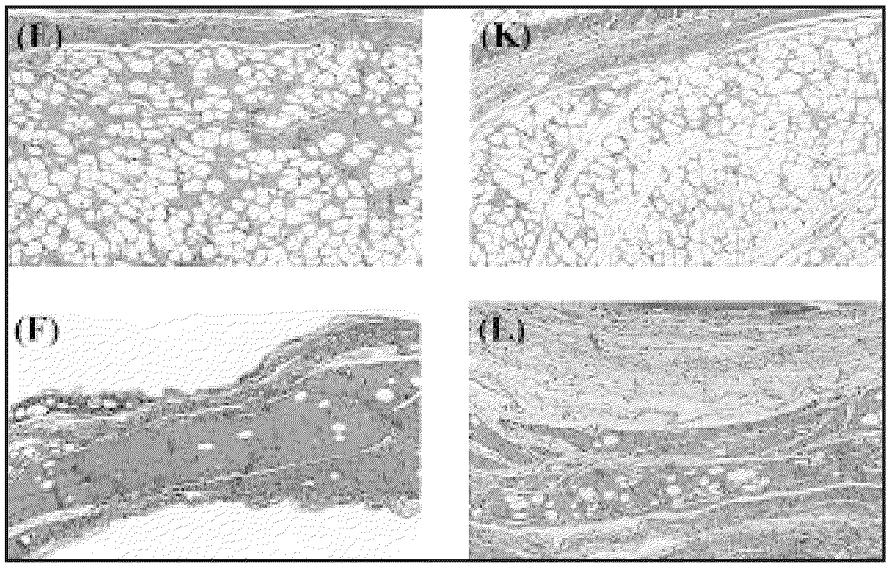
(E)
(K)
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(L)

to evaluate histologically the *de novo* formation of adipose tissue and vascularization. The ratio of the Sudan III-stained tissue area to the whole area of Matrigel implants was measured to express it as the percent adipose tissue. The residual three skin flaps were used to confirm *de novo* adipogenesis by reverse transcription and polymerase chain reaction (RT-PCR) of glycerophosphate dehydrogenase (GPDH) mRNA.

RT-PCR detection of GPDH mRNA in adipose tissue formed de novo

Total RNA was extracted from the tissue mass formed with TRIZOL reagent (Life Technology, GIBCO BRL Products Inc., Rockville, MD). Briefly, 6 weeks after co-implantation of Matrigel with PBS, 0.1 and 1.0 μg of free bFGF, and gelatin microspheres incorporating 0.1 and 1.0 μg of bFGF, the tissue mass formed in the mouse subcutis was carefully collected without contamination of the surrounding tissue. After being minced by a scissors, the tissue was lysed for 7 min at 25°C with TRIZOL reagent. The total RNA was precipitated conventionally with isopropyl alcohol and 70% ethyl alcohol in DDW from the tissue lysate and dried under vacuum.

The prepared total RNA was reverse transcribed to cDNA by First-Strand cDNA Synthesis Kit (Code #27-9261-01, Amersham Pharmacia Biotech Ltd., Tokyo, Japan). DDW containing 5 μg of total RNA (8 μL) was heated at 65°C for 10 min and cooled on ice for 2 min. The RNA solution was mixed on ice with 7 μL of a reverse transcription reaction mixture, which composes of 5 μL of the Bulk First-Strand Reaction Mix, 1 μL of 0.2 μg/μL pd$(N)_6$ primer, and 1 μL of 200 mM dithiothreitol, followed by incubation at 37°C for 60 min and then quickly cooled down on ice.

Oligonucleotides of mouse GPDH primers were purchased from Takara Shuzo Co. Ltd. (Shiga, Japan) and these sequences were as follows: 5′-CTGTGGGGCCTTGAAGAATA-3′ (GPDH, up-stream, sense) and 5′-CCAAGATCGTGGGTAGCAAT-3′ (GPDH, down-stream, antisense). The sense and antisense primers were dissolved in 100 μL of DDW at respective concentrations of 4 μM to prepare a mixed primer solution. The PCR reaction solution was prepared by mixing on ice 81.5 μL of DDW and 18.5 μL of TaKaRa Ex Taq™ reagent (TakaRa Biochemicals, Takara Shuzo Co. Ltd, Shiga, Japan) which contains 8 μL of dNTP Mixture (2.5 mM each), 0.5 μL of 5 U/μL TaKaRa Ex Taq™, 10 μL of 10 × Ex Taq™ Buffer. Then, 1 μL of the prepared cDNA solution was mixed with 2 μL of the mixed primer solution and 7 μL of the PCR solution. The solution mixture was heated at 94°C for 5 min and then subjected to 40 cycles of PCR. One cycle of PCR consisted of 15 s at 94°C, 15 s at 43°C, and 15 s at 72°C.

After the PCR, the amplified products were fractionated by sodium dodecyl sulfate—4.8% polyacrylamide gel electrophoresis (SDS-PAGE) in 90 mM Tris-borate, 2 mM EDTA (pH 8.0). Amplified products were detected by staining with SYBR™ Green I (TaKaRa Biochemicals, Takara Shuzo Co. Ltd) at room temperature for 30 min.

Statistical analysis

All of the data were analyzed by Fisher's LSD test for multiple comparison, and the statistical significance was accepted at $p < 0.05$. Experimental results were expressed as the means ± the standard deviation of the mean.

RESULTS

De novo *formation of adipose tissue and vascularization following treatment of bFGF with or without Matrigel*

Figure 1 shows the tissue appearance of mouse subcutis 6 weeks after treatment with PBS, bFGF-free empty gelatin microspheres, free bFGF, and gelatin microspheres incorporating bFGF with or without Matrigel. When Matrigel was not co-implanted, the appearance of subcutaneous tissue injected with bFGF in the microspheres-incorporated or free form was similar to that of control, PBS-treated mice. A similar result was observed at different bFGF doses (data not shown). Empty microspheres did not affect the tissue appearance. Gelatin microspheres were completely degraded in the tissue, irrespective of the bFGF incorporation. On the contrary, the tissue appearance was greatly influenced by the presence of Matrigel. When bFGF was mixed together with Matrigel for implantion, capillaries were newly formed at the implanted site

of matrigel, although the capillary number was larger for gelatin microspheres incorporating bFGF than for free bFGF. Empty gelatin microspheres did not contribute to vascularization, and the tissue appearance was similar to that of Matrigel alone. The volume of tissue mass formed by co-implantation of Matrigel with bFGF was large compared with that of other implantation groups.

Influence of bFGF dose on the de novo *formation of adipose tissue*

Figure 2 shows the histological sections of mouse subcutis 6 weeks after implantation with the mixture of Matrigel and different amounts of bFGF. Apparently, co-implantation of bFGF formed *de novo* adipose tissue in the implanted site of Matrigel. When the bFGF dose was 0.01, 0.1, or 1.0 μg, gelatin microspheres incorporating bFGF induced ectopic formation of adipose tissue accompanied by capillary formation to a significantly greater extent than that of free bFGF at the same dose. Less formation of adipose tissue was observed at bFGF doses of 0.001 and 10 μg. Especially, 10 μg of bFGF induced inflammatory reaction in the Matrigel implanted site, irrespective of the dosage form of bFGF. No *de novo* formation of adipose tissue was observed at the Matrigel implanted site together with PBS or empty microspheres.

Figure 3 shows lipid staining of subcutaneous sites implanted with the mixture of Matrigel and PBS, empty gelatin microspheres, free bFGF, or gelatin microspheres incorporating bFGF 6 weeks post-implantation. When the histologic sections were stained with Sudan III, matured adipocytes were stained in the tissue mass formed by co-implantation of Matrigel with gelatin microspheres incorporating 0.1 μg of bFGF. A fewer number of stained cells was found in the implant containing a mixture of Matrigel and the same dose of free bFGF. Only a few adipocytes were stained in the tissue mass formed by implantation of Matrigel alone or its mixture with empty gelatin microspheres.

Figure 4 shows the dependence of *do novo* adipogenesis on the bFGF dose. Adipogenesis was assessed by determining the area percentage of Sudan III-stained adipose tissue to the total tissue on histologic sections 6 weeks after co-implantation of Matrigel with bFGF in the incorporated or free form gelatin micro

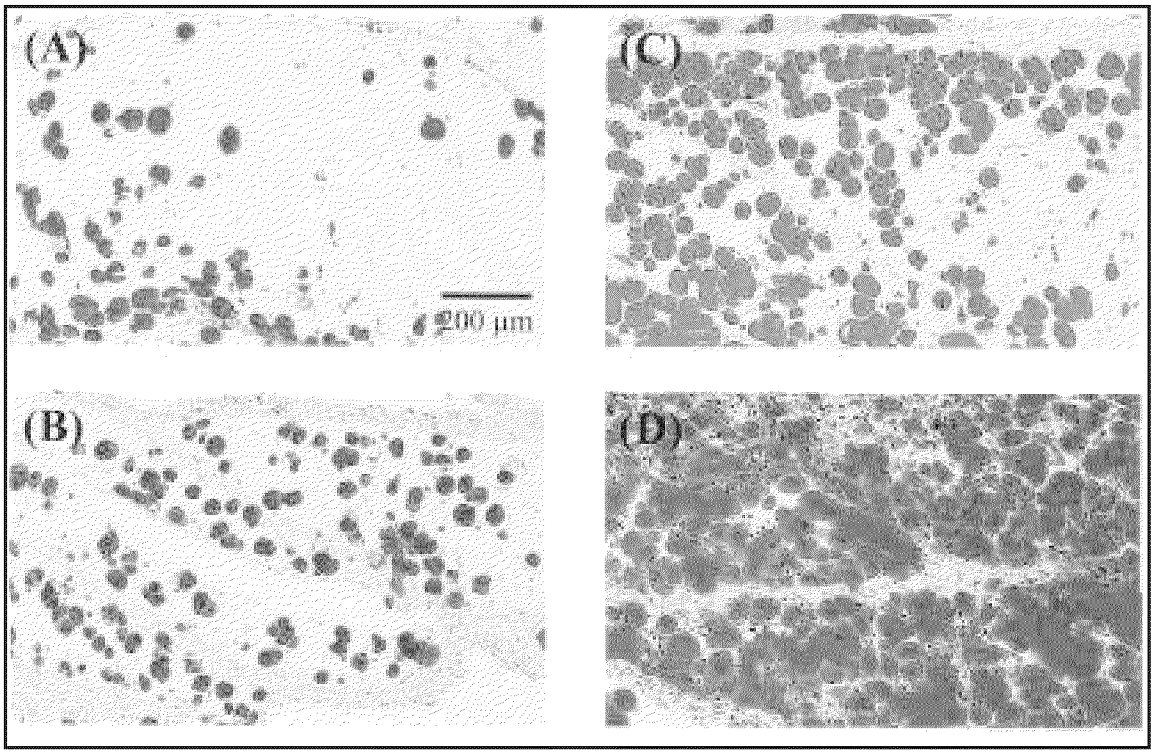

FIG. 3. Lipid staining of mouse subcutis 6 weeks after implantation of Matrigel mixed with PBS **(A)**, bFGF-free, empty gelatin microspheres **(B)**, 0.1 μg of free bFGF **(C)**, and gelatin microspheres incorporating 0.1 μg of bFGF **(D)**. (Magnification: $\times$100, Sudan III staining.)

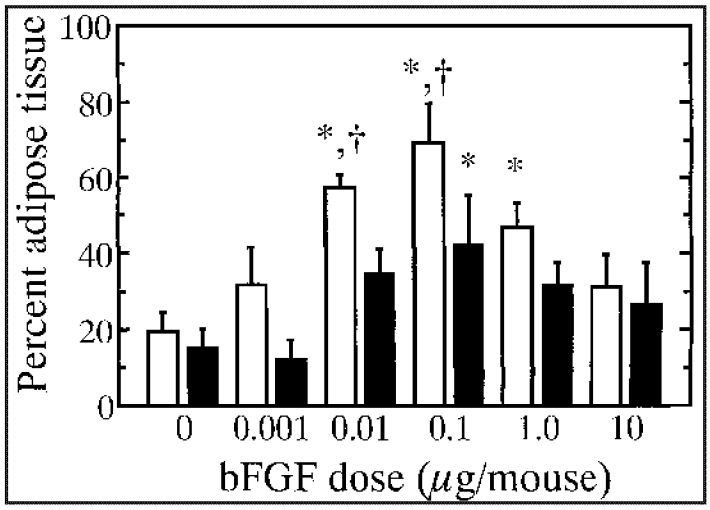

FIG. 4. Effect of bFGF dose on *de novo* adipogenesis 6 weeks after co-implantation of Matrigel with gelatin microspheres incorporating bFGF (□) and free bFGF (■). *, $p < 0.05$ significance against the group implanted with Matrigel mixed with PBS. †, $p < 0.05$ significance against the group implanted with Matrigel mixed with free bFGF at the corresponding dose.

spheres. When gelatin microspheres incorporating 0.01, 0.1, and 1 μg of bFGF were co-implanted with Matrigel, the percent adipose tissue was significantly higher than that of Matrigel alone. On the contrary, for free bFGF, the percent adipose tissue was significantly enhanced at only a dose of 0.1 μg, in contrast to other doses. The highest or lowest dose of bFGF did not enhance adipogenesis and the percent adipose tissue was similar to that of Matrigel alone for both dosage forms of bFGF.

GPDH detection

Figure 5 shows expression of GPDH mRNA products in the tissue mass formed by co-implantation of Matrigel with gelatin microspheres incorporating bFGF or free bFGF. Amplified products of GPDH were not observed when the mixture of Matrigel and PBS was implanted. Co-implantation of Matrigel with gelatin microspheres incorporating 0.1 and 1.0 μg of bFGF induced GPDH mRNA expression, whereas weaker expression was observed for the mixture of Matrigel and 0.1 μg of free bFGF.

DISCUSSION

The hyperplastic formation of adipose tissue in aged animals by feeding with a high carbohydrate or high-fat diet has been investigated intensively. It has been demonstrated in many experiments on rodents that adipose precursor cells possess the potential ability to generate new adipose tissues. The fat depots of mice express large amount of early markers of adipocyte differentiation.[24] A significant population of stromal vascular cells from subcutaneous fat tissues of elderly men and women has been shown to differentiate *in vitro* into adipocytes.[25] Taken together, all of the results suggest that proliferation and differentiation of adipose precursor cells can be promoted depending on their microenvironment. It is recognized that adipocytes and their precursor cells represent only less than one-half of the total cells in adipose tissue; the remaining cells are various blood cells, endothelial cells, and precytes. This tissue cellularity indicates that development of a vascular supply is essential for the generation of maintenance of adipose tissue. What is the microenvironment that allows adipose precursor cells to proliferate and differentiate into matured adipocytes? The present study clearly indicates that such a microenvironment can be provided by implantation of Matrigel together with the release system of bFGF. There will be several reasons to be considered

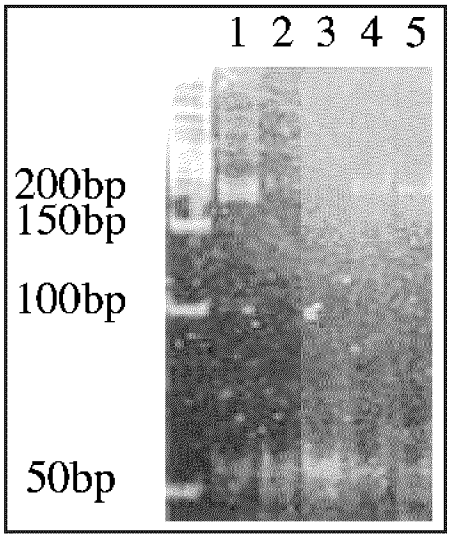

FIG. 5. Expression of GPDH mRNA products of mouse subcutis 6 weeks after implantation of Matrigel mixed with PBS (lane 1), 0.1 μg of free bFGF (lane 2), 1.0 μg of free bFGF (lane 3), and gelatin microspheres incorporating 0.1 (lane 4) or 1.0 μg of bFGF (lane 5).

for the bFGF effect on induced adipogenesis. First, it is possible that the controlled release of bFGF-induced neovascularization, resulting in efficient proliferation and maturation of adipose precursor cells, migrated in the vascularized Matrigel. Second, there is the possibility that bFGF has a direct adipogenic effect. Sheep preadipocytes have been reported to differentiate in a culture medium containing bFGF.[26] It is likely that bFGF increases the number of preadipocytes and the rate of adipocyte differentiation, resulting in enhanced *de novo* adipogenesis.

During the terminal differentiation, adipocytes exhibit marked increases in *de novo* lipogenesis.[27] It is well recognized that the activity level of several proteins and/or mRNA increases with lipogenesis. Among them, GPDH has been used as a representative lipogenesis marker, and the level of activity increase corresponds well with that of preadipocyte differentiation.[27–31] As seen in Fig. 5, it was demonstrated biochemically that the tissue mass formed by the mixture of Matrigel and the gelatin microspheres incorporating bFGF was composed of matured adipocytes.

One promising way to enhance *in vivo* vascularization effectively is to achieve the controlled release of bFGF over an extended period of time. From this point, gelatin microspheres are superior to Matrigel. Significant vascularization was demonstrated to be induced through controlled release of biologically active bFGF from gelatin hydrogel microspheres, in marked contrast to bFGF administered in the solution form.[19,21–23] Histologic examinations revealed that mixing the bFGF-incorporated gelatin microspheres induced neovascularization in Matrigel to a greater extent than free bFGF mixing (Fig. 3). We can be fairly certain that such greater vascularization was one key contributing to significant pronounced formation of adipose tissue (Figs. 4 and 5). Matrigel itself functions to induce angiogenic activity of bFGF to some extent,[14] but the efficacy is not as high as that of the gelatin microspheres. In addition, the adipogenic effect of bFGF should be considered. The bFGF dose dependence indicates that there is an optimal concentration range of bFGF for adipogenesis. It is conceivable that a low dose of bFGF is not enough to exert its angiogenic or adipogenic effect, even though the bioactive protein is released from the gelatin microspheres. Conversely, when the bFGF dose is too high, in addition to the two effects of bFGF, the activity to accelerate infiltration of fibrous tissues into Matrigel will become pronounced. Although adipocyte precursor cells are present in the infiltrated tissue, it is highly possible that the infiltrated fibrous tissue occupies the space in Matrigel necessary for *de novo* formation of adipose tissue.

Another animal experiment revealed that, in place of Matrigel, type I collagen extracted from bovine bone was co-injected subcutaneously into mice together with or without gelatin microspheres incorporat-

ing bFGF. However, none of these treatments induced *de novo* adipogenesis (unpublished data). It is reported that Matrigel enhanced attachment and spreading of predipocytes.[32] These findings indicate that there remains a possibility that Matrigel has additional functions, such as supporting the cell proliferation and differentiation of adipose precursor cells. In an effort to identify adipogenic components of Matrigel, a similar study with implants composed of laminin, type IV collagen, and glycosaminoglycan is currently under way.

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